PEER REVIEWED

Powerful Techniques Promoting Gene Therapy and Gene-mediated Cell Therapy Progress

Gene therapy and gene-mediated cell therapy (e.g. CAR-T cell therapy) approaches hold great promise for treating debilitating diseases with unmet clinical needs. The drive to address the genetic cause of a disorder has brought about a gamut of innovative tools that can correct a cell's function at the DNA level or engineer a cure via *in vivo* or *ex vivo* methodologies. Since 2021, the US Federal Drug and Food Administration (FDA), approved a record number of five gene- and chimeric antigen receptor (CAR)-T cell-therapy products. While there are thousands of clinical trials currently on the horizon, progress of such therapies to the market is hampered by safety, efficacy, and reproducibility concerns.

Market approval relies on consistently presenting robust and reliable data displaying the highest levels of purity, potency (i.e. intended biological activity and/or therapeutic effect), and, ultimately, safety of the products throughout all phases of development. Harnessing the ability of specific techniques, providing accurate and precise data on product characteristics, is key to extending product accessibility to more patients across a wider range of genetic disorders. This article discusses the fundamental principles of gene and gene-mediated-cell therapies, addresses the challenges associated with *in vivo* and *ex vivo* gene delivery approaches, and presents tools the industry can use to support product development.

Over the past two decades, advances in gene therapy and gene-mediated cell therapy have led to the development of versatile treatment strategies which fundamentally involve the delivery of nucleic acids into specific cells using gene delivery vectors. Using an *in vivo* approach, a gene therapy product is delivered directly into a patient, while *ex vivo* gene delivery combines a gene therapy product with allogeneic (patient-derived) or autologous (healthy donor-derived) cells, resulting in engineered cells which can be then transferred into the patient (Figure 1). Depending on the underlying cause of genetic disease, delivery of genetic material into a cell can result in gene augmentation, editing or suppression (Figure 2).

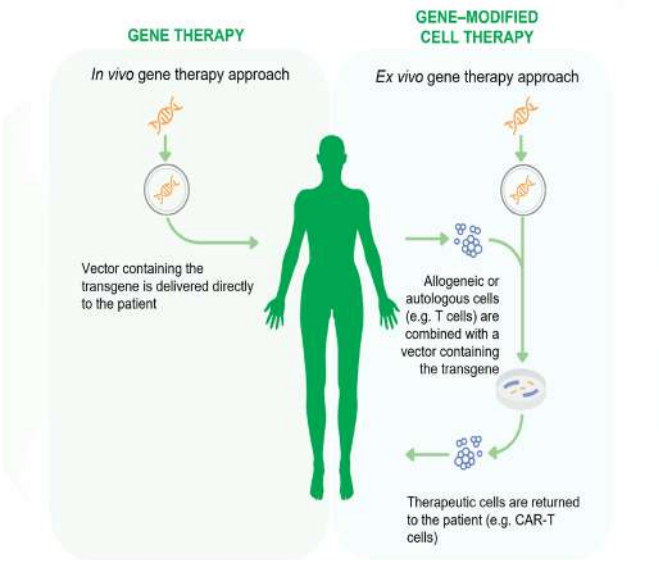


Figure 1. Gene therapy and gene-modified cell therapy approaches

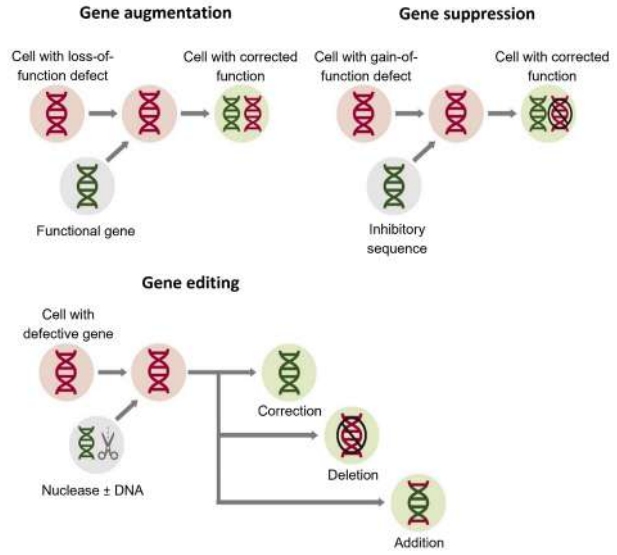


Figure 2. Outcome of gene therapy approaches

Currently, there are two FDA approved gene therapy products (GTPs), both of which are examples of *in vivo* gene augmentation therapy using adeno-associated virus (AAV) vector to deliver functional copies of a gene to alleviate symptoms caused by a dysfunctional protein.[2] Luxturna (Spark Therapeutics), was approved in 2017 for the treatment of biallelic retinal pigment epithelium-specific 65 (RPE65) mutation-associated retinal dystrophy, which can result in blindness.[3] Luxturna can restore normal retinal cell function by supplying an RPE65 transgene thus restoring protein function. Following Luxturna, Zolgensma (Novartis) received marker approval for treatment of spinal muscular atrophy (SMA) in 2019. SMA is the leading inherited cause of infant death resulting primarily from biallelic loss-of-function of survival motor neuron 1 gene (SMN1). Zolgensma works by supplying a functional copy of the SMN gene from which a fully functional protein can be produced. Both clinical trials and post-approval studies assessing the long-term safety and efficacy of Zolgensma treatment demonstrated a significant increase in survival rates and quality of life in infants with the disease.[4,5] Numerous pre-clinical and clinical trials are currently underway exploring how gene editing or suppression can alleviate symptoms of genetic conditions caused, for example, by toxic gain-of-function of proteins, such as in the case of Huntington's disease. Protein overexpression can be treated by gene 'silencing' or by post-

transcriptionally targeting a specific mRNA sequence.[6] Gene editing is a powerful approach that can also be used to restore gene function. Pre-clinical and clinical trials are currently underway, assessing the efficacy of these tools, such as small interfering RNA (siRNA), CRISPR-Cas9 and zinc finger nucleases.[7]

During the process of generating CAR-T cells, an *ex vivo* gene therapy, a CAR is delivered into either autologous or allogeneic T cells via lenti- or retro-viral vectors.[8] This type of gene-mediated cell therapy product (GMCTP) has revolutionised the field of immuno-oncology. Four out of five CAR-T cell therapy products were granted FDA approval in 2022; most recently, Breyanzi (Bristol-Myers) was authorised for the treatment of patients with B-cell lymphoma and works by identifying and eradicating CD19-positive cells, i.e. B cells.[2]

Challenges Associated with Gene Therapy Approaches

Demonstrating purity and potency of a GTP or GMCTP throughout all pre-clinical and clinical stages, and following market approval, is critical to ensuring product efficacy and safety. With gene delivery being a key component of these therapies, use of a safe and specialised gene delivery vector is imperative to product success.

An ideal gene delivery vector would be characterised by its ability to encapsulate a transgene, safely deliver it to a target cell without triggering host immune response and releasing its cargo via endocytosis to either the cytoplasm or nucleus. Viral vectors, commonly used during gene therapy and CAR-T cell therapy development, are costly to manufacture and can come with the risk of insertional oncogenesis (e.g. retroviruses), and high immunogenicity (e.g. adenoviruses) following transduction. AAVs are some of the most extensively researched viruses, and with modified characteristics making them safe to use as gene delivery vehicles, are widely used in clinical *in vivo* gene therapy applications. AAV has unique biological abilities which render it invaluable in the field, such as lack of immunogenicity and pathogenicity in humans. Engineered AAV vectors used in gene therapy, lack 96% of the viral genome and are essentially recombinant protein-based shells unable to replicate or integrate into the human genome.[9]

Due to their intrinsic characteristics and versatility of applications, AAVs are likely to remain the leading tools for *in vivo* gene therapies for years to come. Despite their potential, AAV-mediated gene therapy is accompanied by safety and efficacy concerns raised during the manufacturing and clinical process. During the initial steps of both *in vivo* and *ex vivo* gene therapy development, confirmation of the identity, integrity and purity of the viral vector and assessment of viral titre is critical and will impact the success the final product. During the product purification phase, contaminants derived from common cell culture procedures, such as residual host DNA, bacteria or other viruses, can be present in the final batch posing a significant health risk to the patient and must be removed.

Finally, the manufacturer needs to verify therapeutic delivery of the transgene into the target cell and assess biological effect by measuring its expression. *Ex vivo* gene therapy that uses genome integrating retro- or lenti-viruses for cell transduction can provide long-term therapeutic benefits through prolonged transgene expression. Viral vector genome integration is, however, typically associated with a higher risk of insertional oncogenesis10. Addressing such concerns, the FDA recommends that GMCTPs are accompanied by transgene integration and transduction efficiency studies. Specifically, the integration copy number should be no more than five copies per cell to minimise any health risks, while still ensuring a potent therapeutic product at lot release.[10]

Techniques Assisting Therapeutic Product Development

Using reliable techniques capable of addressing all the challenges during GTP and GMCTP development is essential for ensuring product safety and efficacy. SDS-PAGE followed by protein staining and western blotting is commonly used to validate both viral vector purity and identity. Using stain-free protein gels assist in expediting this process and enable rapid estimation of the capsid protein ratios in viral samples.[11] Western blotting can also be used to verify product identity and assess transgene protein expression during early-stage product development, while flow cytometry, can be employed to detect protein expression on target cells during pre-clinical or clinical stages via high-throughput, high-speed cell phenotyping.

One of the most established techniques commonly used to measure transgene or viral messenger RNA (mRNA) expression is reverse transcription quantitative PCR (RT-qPCR).[11] A one-step RT-qPCR approach combines the reverse transcriptase and quantitative PCR steps in a single reaction, resulting in reduced sample manipulation and faster data acquisition. Alternatively, if the cDNA needs to be isolated and used for any downstream experiments or reserved for auditing purposes, a two-step RT-qPCR approach should be taken, whereby the two processes are separated. Depending on the manufacturer's objectives, each method has its own merits; the two-step approach offers higher sensitivity and efficacy, while the one-step approach can generate data at a faster rate with less external interference.[12]

Application of Droplet Digital PCR Throughout Development

Real-time quantitative PCR (qPCR) utilises a standard curve to estimate nucleic acid quantities. Although widely use, this method has inherent limitations with reliable data acquisition being susceptible to template quality, reaction conditions and sample variability. Compared to real-time qPCR, droplet digital PCR (ddPCR) delivers higher rates of reproducibility, offering absolute nucleic acid quantification across the lifecycle of a product (Figure 3). Crucially, ddPCR does not rely on a standard curve or perfect DNA amplification efficiency, meaning significantly less variation compared to real-time qPCR. As such, ddPCR is deemed the 'gold standard' for measuring viral titration within the gene and gene-mediated-cell therapy field.[13]

Utilising a water-in-oil droplet system and a single low-volume sample, ddPCR counts nucleic acid molecules partitioned in over 20,000 individual volumetrically defined droplets, each serving as individual PCRs. Each droplet is then analysed to determine the fraction of PCR-positive droplets in the original sample. The concentration of target DNA template in the original sample can then be calculated using Poisson statistics. This method is suitable for the analysis of samples throughout GTP and GMCTP development.

During 'in-process' testing, ddPCR can be used to assess plasmid quality prior to transfection, and precisely measure viral vector titre during the virus production and recovery process – all of which are vital for determining therapeutic efficacy downstream. Through the final quality assurance / quality control steps, ddPCR can confirm potency of the purified viral titre, and assure sample safety through reducing mycoplasma false positives and detecting residual host DNA. Beyond the manufacturing process, and into the clinic, ddPCR can be used to verify the therapeutic delivery of the GTP or GMCTP and assess transgene expression.

Safeguarding Therapeutic Potential

There are currently over 2400 cell and gene therapy trials taking

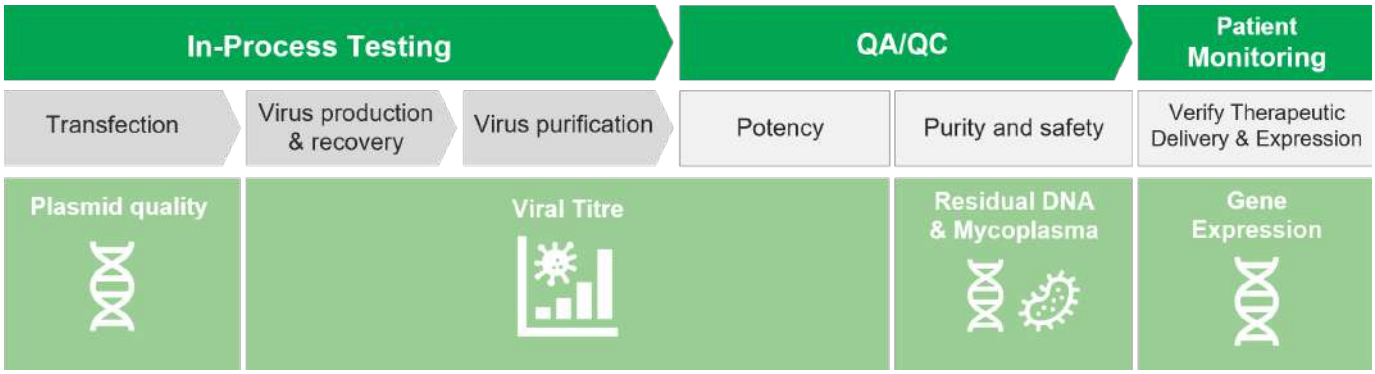
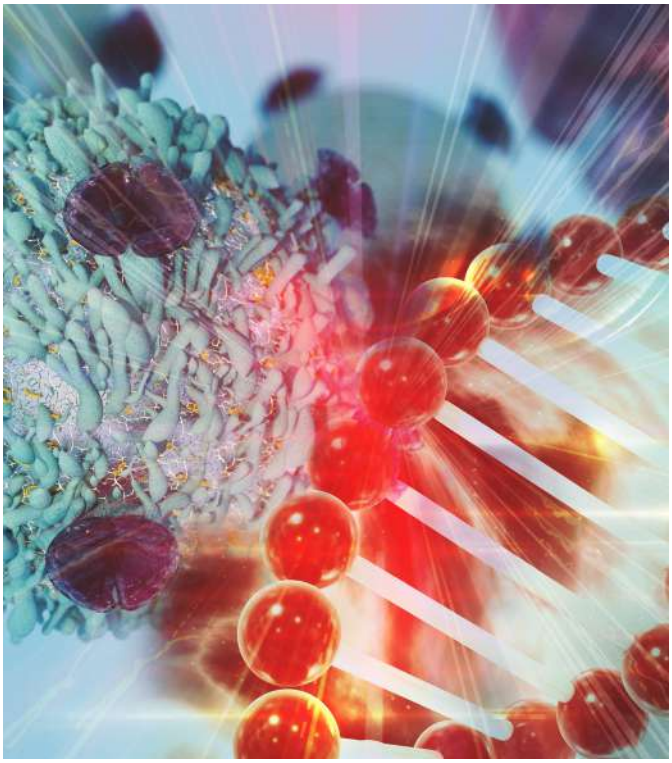


Figure 3. Application of ddPCR in gene therapy approaches

place across the globe, with rare cancers being the main target for treatment14. Novel therapeutic modalities have undoubtedly contributed to the growing number of products entering the market every year targeting diseases with unmet clinical needs. Despite the promising future ahead, the progress of such therapies is stalled by safety and reproducibility concerns.

Downstream efficacy and safety of any GTP or GMCTP relies heavily on consistently demonstrating purity and potency throughout earlier stages of development and following lot release. Collaboration and transparency between regulatory bodies, GTP/GMCTP manufacturers, and tool or reagent providers is imperative to the development of such life-changing therapeutics.

Specifically, reproducible data quantification and reliable product verification requires tools that comply with regulatory requirements. Adhering to a set of stringent, product-specific standards, that fulfil applicable regulatory requirements associated with medical devices, such as ISO 13485, will guarantee the highest levels of quality when it comes to product development. Moreover, data must be documented electronically and stored securely to ensure it is readily available for auditing, as outlined in the US FDA Code of Federal Regulations (CFR) Part 11. Making certain these processes are in place from the start, can aid in providing access to reliable and trustworthy data throughout therapeutic development and manufacturing, maximising the chance of success.



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